Functional Hydrogel – Biomineral Composites Inspired by Natural Bone

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A urea-mediated mineralization technique was developed to enable the formation of pHEMA-based hydrogel-calcium phosphate composites with excellent polymer-mineral interfacial adhesion strength that is desirable for bone mimics. This mineralization method was also applied to generate more sophisticated composites containing functional hydrogels that possess anionic groups mimicking the extracellular matrix proteins in bone.

Introduction

Bone is a hierarchical composite material that consists of carbonated calcium phosphates (CP) and protein matrices. The active organic matrix of mineralized tissues typically consists of a structural component (e.g. type I collagen) and acidic mineral nucleating proteins (e.g. bone sialoprotein rich in glutamate, or phosphophoryn rich in phosphoserine and aspartate).[1-3] They

play essential roles in defining both the highly efficient nucleation (and subsequent high-affinity mineral integration) process and the toughness of bone. Matrix-mediated nucleation in nature is believed to occur by an epitaxial mechanism, in which a lattice match between the anionic organic matrix and the nascent crystal lowers the interfacial freee-energy barrier to critical nucleus formation.[2] In addtion, the presence of protein matrices also provides the necessary toughness complementary to the strong yet brittle mineral composition. Unlike the apatite crystals that cannot dissipate much energy, the 3-dimensional macromolecular network provides toughening mechanisms, such as divalent cation-based ionic bridges between two anionic sites on the protein scaffold, to deter crack propagation.[4]

The development of a new generation of bone-like composite materials with improved mechanical properties and enhanced biocompatibility calls for a biomimetic synthetic approach using natural bone as a guide. Our strategy involves the generation of functional polymer scaffolds displaying surface ligands that mimic critical extracellular matrix components of bone, and their high-affinity integration with biominerals. Specifically, these functional polymer scaffolds are derived from poly(2-hydroxyethyl methacrylate), or pHEMA, which is known to be biocompatible and closely mimic the hydrogel-like property of collagen. The polymerization of HEMA and its co-monomers is water compatible, allowing incorporation of anionic ligands that mimic the acidic matrix proteins regulating mineral growth,[2,3] and biological epitopes such as the tripeptide RGD[5] that promote cellular adhesion. The general assembling strategy of pHEMA-based functional hydrogel network and the biomimetic ligands copolymerized with HEMA are illustrated in Figure 1.

Here we introduce a novel mineralization method that leads to rapid, high-affinity integration of calcium phosphate with pHEMA-based hydrogels, a key step in the fabrication of functional bone-like composites.

Experimental

Hydrogel preparation. pHEMA and its copolymers containing various anionic residues were prepared via radical polymerization as previously reported.[6] The formed gels were washed extensively with water to ensure the complete removal of unreacted monomers before they were used for mineralization and further physical characterizations.

Mineralization of hydrogels with the urea-mediated process. Hydroxyapatite, or HA, (2.95 g) was suspended into 200 mL of Milli-Q water with stirring, and 2 M HCl was added sequentially until all the HA suspension was dissolved at a final pH of 2.5-3. Urea (24 g) was then dissolved into the

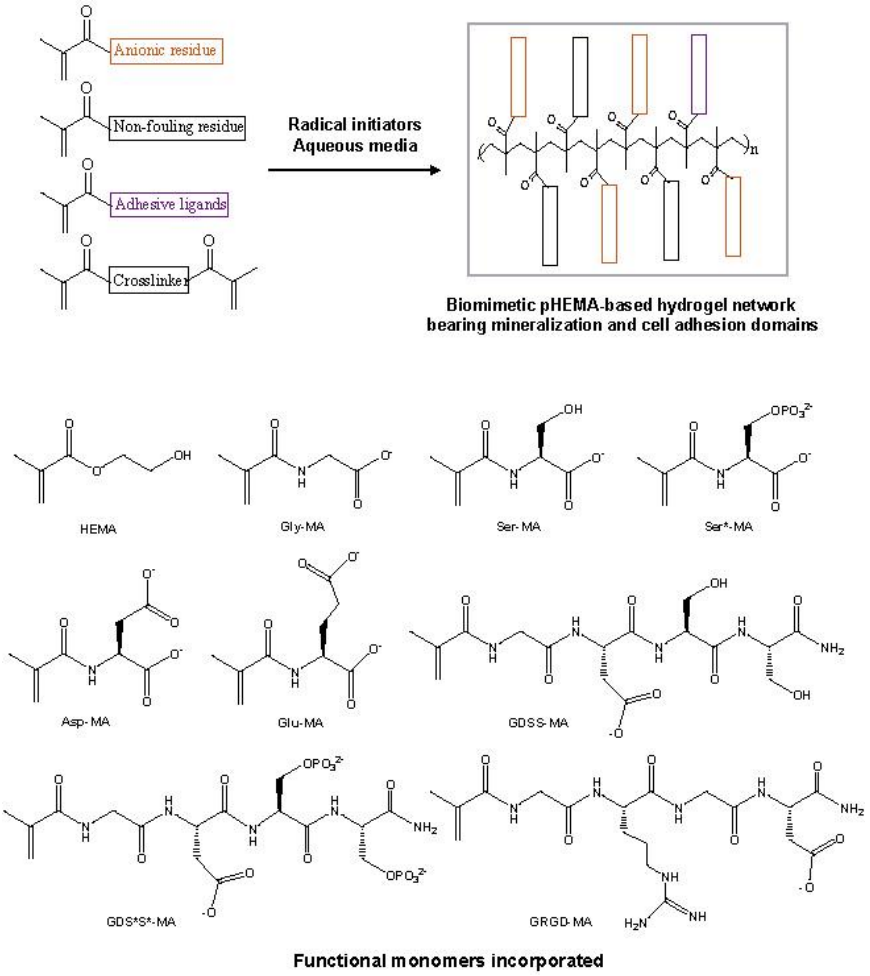

Figure 1. Assembling of a hydrogel scaffold containing multiple functional domains (top) and the anionic or adhesive monomers to be copolymerized with HEMA (bottom). *Note that the anionic monomers differ in both the type of anionic residues and the number of anionic charges carried per side chain.*

solution to reach a concentration of 2 M. Each hydrogel strip was then immersed into the acidic HA-urea stock solution. The solution was heated to 95 ºC with varied linear heating rates (0.1 to 1.0 ºC/min) without agitation of the mineralization solution and maintained at that temperature overnight when necessary (for preparation of composites with thicker CP layers).

SEM-EDS. All SEM micrographs of freeze-dried hydrogels and hydrogel-mineral composites were obtained with a ISI-DS 13OC dual stage SEM with associated EDS. Samples were either coated with Au or Pt on a BAL-TEC, SCD 050 sputter coater to achieve optimal imaging results, or coated with carbon for EDS analysis.

XRD. The crystallinity of the mineral phase of the composites was evaluated by XRD with a Siemens D500 instrument using Cu Kα radiation.

Evaluation of mineral-hydrogel interfacial adhesion. In order to evaluate the adherence of the mineral layers attached to pHEMA hydrogels, the relative crack resistance was qualitatively evaluated by indentation. The indentation test was performed on the freeze-dried composite using a Vickers indentor (Micromet, Buehler, Ltd., USA). Loads from 5 to 15 Newtons were applied for 20 seconds for each measurement. After indentation, the samples where analyzed by SEM in order to check for delamination.

Results and Discussion

Calcium apatites are known to promote bone apposition and differentiation of mesenchymal cells to osteoblasts.[7] In this work, synthetic HA was used in the fabrication of hydrogel-based bonelike composite materials. HA has limited solubility in water at neutral and basic pH but is highly soluble at acidic pH.[8] Based on this property, we devised a urea-mediated solution precipitation technique, in which a segment of pHEMA hydrogel was soaked in an acidic solution of HA containing a high concentration of urea. Upon gradual heating (without stirring) from room temperature to 95 ºC, urea started to decompose and the pH slowly increased (around pH 8). Under these conditions, some hydrolysis of the 2-hydroxyethyl esters occurred, promoting heterogeneous nucleation and 2-dimensional growth of a thin CP layer at the hydrogel surface.[6]

The strong affinity between calcium and the *in situ* generated acidic surface of pHEMA led to the 2-dimensional outward growth and eventual merge of CP from individual nucleation sites (the bright centers indicated by arrows in Fig. 2A & 2B). The calibrated EDS area analysis performed on the mineral surface of the composite revealed a Ca/P ratio (1.6±0.1) similar to that of synthetic HA (Fig. 2C). X-ray diffraction (XRD) analysis performed on the mineralized pHEMA composite indicated that the CP layer was either nanocrystalline or amorphous (data not shown). The adhesion strength of the CP layer to the gel surface was qualitatively evaluated by microindentation analysis performed on the surface of the freeze-dried hydrogel-CP composite. No delamination of the mineral layer was observed by SEM (Fig. 2D) even after Vickers indentations

with loads up to 15 N, an indication of good adhesion at the mineral-gel interface. This represents a major improvement over the widely used simulated body fluid mineralization method, which results in flake-like crystal apatite coatings that tend to delaminate easily upon drying.[9]

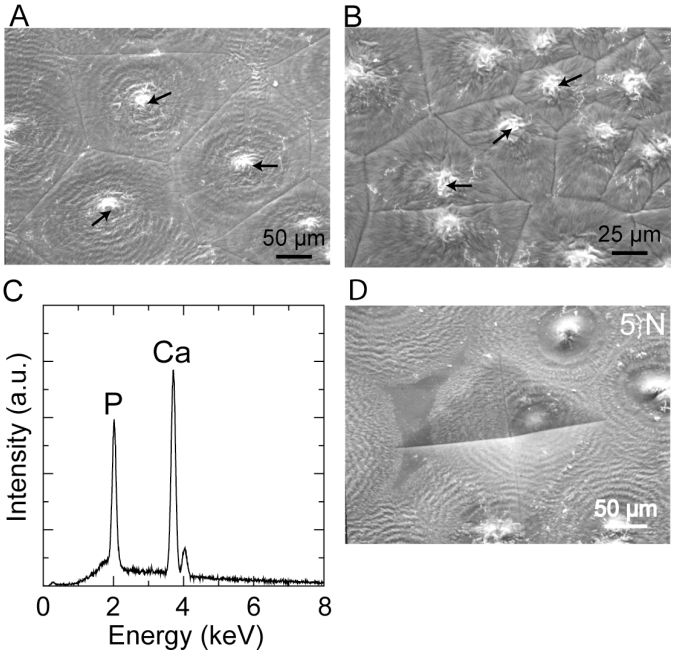

Figure 2. Morphology, chemical composition and microindentation analysis of calcium phosphate layer grown on the surface of pHEMA via the urea-mediated process. *The mineralization process lasted for 2 hours, with an average heating rate around 0.6 °/min. (A) & (B): SEM showing different patterns of 2-dimensional circular outward growth of mineral layers from multiple nucleation sites (indicated by arrows) on the acidic surface of pHEMA. Note the full coverage of the hydrogel surface with mineral layers and the sharp edges separating neighboring domains. (C): SEM-associated EDS area analysis of the mineral layer shown in micrograph A, confirming the chemical composition and Ca/P ratio that is typical for HA. (D): SEM showing an indent formed on the surface of mineralized pHEMA using a Vickers microindenter with a load of 5 N. The mineral layer did not delaminate.*

Several notable features of this mineralization procedure include: 1) increasing pH and temperature during the process promotes the hydrolysis of the ethyl ester side chains of pHEMA and leads to the *in-situ* generation of an acidic surface and a partially acidic interior that has high affinity for calcium

ions; 2) the high affinity between the calcium ions and the exposed carboxylate groups at the gel surface translates into a low interfacial energy between the hydrogel and calcium phosphate, and consequently, a low energy barrier for the heterogeneous nucleation of mineral on the hydrogel surface; 3) the thermo-decomposition of urea allows a homogeneous variation of pH across the solution, avoiding a sudden local pH change that is commonly observed with strong base-induced heterogeneous precipitation.

We also investigated how external factors, such as heating rate, the agitation of the mineral stock solution and the duration of the process, may affect the outcome of the gel-CP composite formation.[10]

We first demonstrated that avoiding direct stirring of the HA-urea mineral stock solution, which promotes homogeneous precipitation of HA across the solution, is essential in achieving the desired heterogeneous nucleation and growth of CP on the *in situ* generated acidic gel surface.

We then applied a range of linear heating rates (1.0 ºC/min to 0.1 ºC/min, from room temperature to 95 ºC) to prepare the gel-mineral composites. We showed that a relatively fast heating rate such as 1.0 ºC/min did not lead to a level of mineralization of the pHEMA gel that was detectable by either SEM or the associated EDS analysis (data not shown). When a 0.5 ºC/min heating rate was applied, the formation of circular mineral layers on the hydrogel surface was observed (Fig. 3A), with similar 2-dimensional outward growth pattern formed around individual nucleation sites. When the linear heating rate was lowered to 0.2 ºC/min, the surface of the hydrogel-mineral composite was fully covered with well-merged circular CP layers (Fig. 3B), suggesting that a slower heating rate and a more sufficient overall mineralization time promote the formation of better-merged CP layers on the gel surface. The most pronounced feature resulted from the slowest linear heating rate attempted (0.1 ºC/min), however, was the dramatic increase of the number of nucleation sites formed on the pHEMA hydrogel surface (Fig. 3C). Longer exposure of the pHEMA gel to any given pH during the urea-mediated process is likely to lead to a more sufficient hydrolysis of the ethyl ester side chains, resulting in increased numbers of surface carboxylates that could serve as tight calcium ion binders and initial nucleation sites.

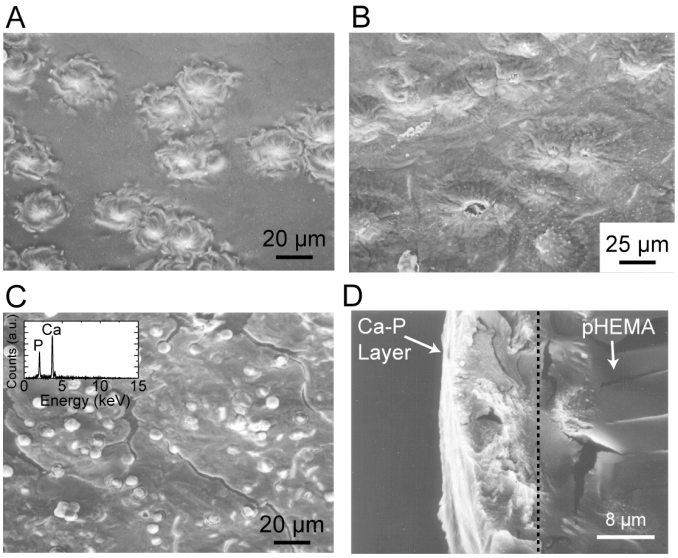

Figure 3. Effects of varied heating rates and duration of the urea-mediated mineralization process on the formation of pHEMA-CP composites. *(A): SEM showing 2-dimensional outward growth of mineral rings from multiple nucleation sites with a linear heating rate of 0.5 ºC/min. (B): SEM showing 2-dimensional circular outward growth and merge of mineral layers from multiple nucleation sites with a linear heating rate of 0.2 ºC/min. Note the full coverage of the hydrogel surface with the mineral layer. (C): SEM showing the full coverage of mineral layers on the surface with a linear heating rate of 0.1 ºC/min. Note the overwhelming amount of nucleation sites scattered throughout the merged mineral domains. The inset shows the EDS area analysis performed over the same fully mineralized surface, confirming the chemical composition and Ca/P ratio that is typical for HA. (D): SEM showing the view of a cross-section of the pHEMA-mineral composite after extended mineralization (10 h after reaching 95 ºC). The sample stage was tilted 45º. Note the micron scale thickness of the mineral layer and the fine integration at the mineral-gel interface.*

Finally, we examined the possibility of forming thicker CP layers over the pHEMA gel surface by extending the mineralization time for another 10-12 hours after reaching 95 ºC. Mineral coatings with thicknesses up to several microns were obtained, with good integration at the mineral-gel interface as shown in a cross-section image of the composite (Fig. 2D).

This urea-mediated mineralization strategy can be extended to other calcium phosphates and pHEMA based functional hydrogel copolymers. The in vivo resorption rates of calcium phosphates vary greatly. For instance, crystalline hydroxyapatite (HA) is hardly soluble and its resorption could take years while tricalcium phosphate is more soluble and its resorption typically occurs in months.[11] The mineralization method we developed can be applied to a range of calcium phosphates (CPs) to produce hydrogel-CP composites with tunable in vivo bio-resorbability. Further, as shown in Figure 4, when the method was applied to the mineralizaton of copolymers of HEMA containing anionic residues that mimic the typical acidic sequences in bone sialoprotein (BSP) or phosphophoryn in dentin, similar mineralization patterns were observed (Fig. 4). Two-dimensional circular mineral growth and the saturation of the gel surface with amorphous CP layer preceded the surface-independent growth of crystalline calcium apatite. As shown in Figure 4B, during fracture of the composite, the CP layer did not delaminate, again suggesting excellent adhesion at the gel-apatite interface.

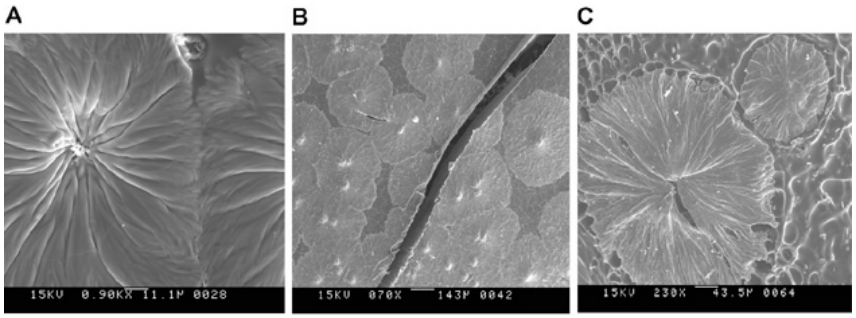

Figure 4. A urea-mediated mineralization process leads to high-affinity growth of CP on pHEMA-based hydrogel copolymers displaying various anionic residues. *Hydrogels mineralized here were pHEMA with 5% Glu-MA (A), 5% Gly-MA (B) and 5% Ser-MA (C). Note that the deliberate fracturing of the composite (B) did not lead to delamination of any circular CP domains, suggesting an excellent gel-mineral interfacial adhesion strength.*

It is worth noting, however, the distinction of subtle differences between the effects of various anionic residues (shown in the bottom panel of Figure 1) on urea-mediated mineral growth patterns is difficult using the pHEMA-based scaffold. The in situ hydrolysis of the 2-hydroxyethyl side chains of pHEMA exposes new surface carboxylates that are powerful competing mineral-nucleators. This issue is now being addressed by copolymerizing biomimetic anionic methacrylamide monomers with an analog of HEMA that is resistant to

hydrolysis under the urea mediated mineralization condition. The new system allows us to investigate the role individual anionic residue plays in template driven mineralization without the interference of competing mineral nucleators generated in situ during the urea-mediated thermal treatment.

Finally, the pHEMA-based functional hydrogel–CP composites developed here are subjects of extensive in vitro and in vivo evaluations. Our preliminary in vitro cell culture evaluations have shown that osteoblasts can adhere and proliferate over the functional hydrogel-CP composites containing up to 10% anionic residues. We are also interested to learn how the underlying anionic mineral binding motifs of the composite would direct new mineral deposition by osteoblasts once the amorphous (or nanocrystalline) CP layer is resorbed by osteoclasts upon implantation.

Conclusions

We have developed a novel template-driven nucleation and mineral growth process to enable high-affinity integration of calcium phosphate with pHEMA-type functional hydrogel scaffolds. This mineralization technique exposes carboxylate groups on the surface of crosslinked pHEMA or its copolymers displaying biomimetic mineral nucleating ligands, promoting high-affinity nucleation and growth of CP on the gel surface. It provides a foundation for integrating template-driven biomineralization with the versatile properties of 3-dimensional hydrogel scaffolds, and opens the door for application of pHEMA in the design of functional bone-like composites.

Acknowledgements

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